ENHANCING LEARNING AND MEMORY IN THE AGED: INTERACTIONS BETWEEN DIETARY SUPPLEMENTATION AND EXERCISE

BY

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THESIS

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Abstract

Age associated cognitive decline in human and rodents has been linked with decreases in hippocampal neurogenesis and a chronic low grade inflammation in the central nervous system. Physical exercise increases neurogenesis and reverses some cognitive deficits observed in the elderly, but the extent to which dietary supplementation may interact with exercise is unknown. Epigallocatechin gallate (EGCG, a catechin high in green tea) can ameliorate age-related cognitive decline. In addition, β-alanine (β-Ala, a naturally occurring amino acid) may be beneficial against cognitive aging. We hypothesized that exercise (voluntary wheel running, VWR) and dietary supplementation with EGCG (182 mg/kg/d) and β-Ala (417 mg/kg/d) would interact to improve cognition of aged mice. Balb/c mice aged (19 mo) served as sedentary controls or were provided access to running with or without EGCG + β-Ala for 4 weeks. The Morris water maze (MWM) and contextual fear conditioning (CFC) were used to assess learning and memory while BrdU labeling was used to measure new cell proliferation in the dentate gyrus (DG). VWR improved cognition during probe trials in the MWM, increased time spent frozen during both context and auditory cue testing in CFC, and increased BDNF mRNA expression within hippocampus. Dietary supplementation did not affect any of these measurements. Collectively, these data verify that exercise has positive effects on cognition of aged mice by enhancing newborn cells in the DG and increasing neurotrophin expression.

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Chapter 1

General Introduction and Justification

The 85 and older group is the fastest growing segment of the US population and is projected to account for 4.3% of the total population by 2050 (U.S. Census Bureau, 2008). The cost of dementia and related cognitive impairments was $203 billion in 2013 and is expected to soar to $1.2 trillion by 2050 in the United States alone (Alzheimer ’s & Dementia, 2013). Age-related neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), and amyotrophic lateral sclerosis have increased at alarming rates paralleling the increase in prevalence of the aged population. Many brain pathologies, including those mentioned, result in loss of neurons (a largely irreversible outcome). In contrast, normal aging results in neural dysfunction associated with increased oxidative stress, elevated levels of inflammatory markers, decreased volume of hippocampi, and diminished production of new neurons within the hippocampus (Erickson et al., 2011; Frank-Cannon et al., 2009; Joseph et al., 2005; Kuhn et al., 1996). Cognitive decline has been attributed to these deleterious consequences of aging. Cognitive aging is a decrease in normal general cognitive functioning associated with increasing age (Salthouse, 1991). These non-pathological changes associated with cognitive aging are not necessarily permanent and thus may be prevented or even reversed. Thus, it is reasonable to consider dietary and/or lifestyle interventions that promote healthier aging leading to a reduced burden on global healthcare systems.

Physical activity and dietary supplementation have been shown to affect several parameters of age-related cognitive decline under various experimental conditions (Artioli et al., 2010; Assuncao et al., 2011; del Favero et al., 2012; Gomes da Silva et al., 2013; Jia et al., 2013; Kohman et al., 2012; Kramer et al., 2006; van Praag et al., 2005; Wang et al., 2012). Since diet

and exercise interventions can coexist it is possible that they have complementary effects on the brain. Ergo, the concurrent study investigated the effects of exercise and dietary supplementation, alone and in combination, on learning and memory and molecular markers known to impact cognition in aged mice.

The most prominent intervention to combat aging-associated maladies is aerobic exercise (Ahlskog et al., 2011). Exercise increases cognitive performance across various training methods, types of cognitive task, or participant characteristics (Colcombe and Kramer, 2003). The cognitive benefits of exercise include the induction of genes associated with neuron plasticity, an increase in brain vascularization, and enhanced neurogenesis (Cotman and Berchtold, 2002; van Praag, et al., 2005). Exercise also has a beneficial role in tempering the immune system. Exercise has been shown to modulate immune activity in the central nervous system (CNS; e.g. reducing microglia interleukin(IL)-1β mRNA expression [Barrientos et al., 2011]) increasing mRNA expression of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor 1 (IGF-1) (Cotman and Berchtold, 2007; Kohman et al., 2012; Woods et al., 2002). Additionally, exercise enhances neurogenesis in the hippocampus which is associated with improved learning and memory (Kohman et al., 2012; van Praag et al., 2005). Thus, engaging in aerobic exercise may be a powerful lifestyle intervention that can augment and maintain cognitive function across the lifespan (Bielak, et al., 2014).

Similarly, epigallocatechin-3-gallate (EGCG), found at high levels in green tea, has gained considerable attention in the scientific community and in mainstream media for its benefits in human health. Epidemiological studies have linked a higher consumption of green tea with lower prevalence of cognitive impairment in humans (Kuriyama et al., 2006). EGCG is believed to improve vascular function, increase antioxidant capacity, and decrease inflammation

(Cabrera et al., 2006). EGCG may protect against age-related cognitive decline because of its neuroprotective role (Joseph et al., 1999; Mandel and Youdim, 2004; Vauzour et al., 2008; Williams et al., 2008).

In addition, β-alanine (β-ala) may have potential health benefits in the older adult population (del Favero et al., 2012). Dietary β-ala has been shown to increase neurotrophin levels in brain, in particular, BDNF protein in the hippocampus (Murakami and Furuse, 2010). Although the precise mechanism by which β-ala supplementation exerts its beneficial effects on muscle and brain are unknown, the effects of β-ala may be mediated through its contribution to carnosine synthesis. β-ala is the rate limiting precursor for carnosine synthesis and has the ability to attenuate fatigue by enhancing intramuscular buffering capacity. Research examining the effects of carnosine has demonstrated its potential to improve or delay the onset of neurodegenerative disorders induced by oxidative stress and inflammation (Boldyrev et al., 2010; Corona et al., 2011; del Favero et al., 2012). However, carnosine has limited absorption from the gut, thus β-ala is likely a more realistic mechanism for increasing the endogenous levels of carnosine while also adding the as yet unknown benefits directly attributed to β-ala (Hoffman et al., 2012).

Lifestyle interventions and dietary supplements that attenuate inflammation and oxidative stress may be cheap and efficient means to improve cognition in aged individuals. Although exercise and dietary supplements are typically researched independently of one another, it is likely that a combination of exercise and dietary supplements could enhance cognitive function in the aged in a synergistic manner. Therefore, this research examined the effects of aerobic exercise and diet supplementation with EGCG and β-Ala on learning and memory in aged mice.

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Chapter 2

Literature Review

2.1 Characteristics of Aging

Aging is associated with an increased risk of chronic health concerns that impact cognitive function and quality of life in the elderly. Neurodegenerative disorders are of particular concern in aging populations. It is estimated that the incidence of dementia will double every 20 years, effecting nearly 81 million people by 2040 (Ferri et al., 2005). Furthermore, the prevalence of AD, the most common form of dementia known for its devastating effects on memory and learning, continues to rise along with the increase in the aged population. However, a decline in learning and memory function is not restricted to persons affected by neurological disease and in fact may be present during healthy aging (Braver et al., 2001; Prince et al., 2011). The hippocampus, known for its role in memory and learning, undergoes morphological and biochemical changes that affect “declarative” or “explicit” memory and spatial learning (Geinisman et al., 1995). Indeed, inflammatory processes, which also increase with old age decreases neurotrophic support of hippocampal cells resulting in poor cognitive performance (Di Filippo et al., 2013; Griffin et al., 2006; Katsuki et al., 1990).

Systemic Changes in Immune Function Associated with Increased Age

The brain was once thought to be an “immune-privileged” organ, but recent evidence has demonstrated that the brain is responsive to systemic immune cues and has resident immune cells. Microglia are the primary immune-competent cell in the CNS but astrocytes, pericytes and perivascular macrophages all contribute to the immune response in brain. (Galea et al., 2007).

Importantly, the CNS and the immune system maintain bi-directional communication via neural and humoral pathways. This communication is vital for mounting an appropriate physiological and behavioral response during infection and subsequently suppressing the immune response when infection is controlled (Dantzer et al., 1998). Proinflammatory cytokines produced in the periphery in response to infection or injury act on the brain following passive or active transport across the blood brain barrier (BBB). Cytokines that are transported across BBB or diffuse into circumventricular organs activate microglia cells that then produce proinflammatory mediators within CNS tissues or alternatively from endothelial cells that form the BBB (Banks et al., 2001; Maier, 2003; Reyes et al., 1999). In normal healthy brain, activation of brain immune cells is tightly regulated by neuronal control to limit the potentially damaging effects that may occur from excess production of inflammatory mediators that increase oxidative stress.

A progressive increase in proinflammatory status accompanied with a global reduction in the capacity to correctly respond to a variety of stressors are major characteristics associated with increased age (Franceschi et al., 2000). Old age is associated with dysregulated inflammation both in the basal state and during systemic immune challenges that leads to greater immune activation in response to immune stimulus. Activation of the immune system in old mice (20 months and greater) and humans over the age of 65 years can lead to a persistent central and peripheral inflammatory state (Frank et al., 2006a; Godbout et al., 2005; Streit et al., 2004; Weaver et al., 2002). This scenario is usually exacerbated when coupled with chronic inflammatory conditions such as AD, PD, or chronic peripheral inflammatory disease such as atherosclerosis and diabetes (Blalock et al., 2003; Ferrari and Tarelli, 2011; Holmes et al., 2009). Inflammation in the brain, termed neuroinflammation, is considered a mediator triggering increased oxidative stress and decline in cognitive function, specifically learning and memory.

Free Radical theory of Inflammation and Aging

Normal brain aging is associated with an increase in proinflammatory cytokines and oxidative stress. Accumulation of free radical damage as a result of cellular senescence in brain leads to a chronic increase in the expression of inflammatory mediators (Godbout et al., 2005; Lee et al., 2000; Nicolle et al., 2001). This combination of inflammation and oxidative stress increases apoptotic neuronal cells in neurodegenerative diseases. Age-associated changes in oxidative stress leads to increased production of free radicals such as reactive oxygen and nitrogen species in brain. This disruption in the normal environment and function of the brain includes a loss of trophic support from microglia and astrocytes that leads to a greater loss of neurons (Ferrini et al., 2001). In response to neuronal loss and increased oxidative stress, microglia become more reactive likely due to loss in restraint from neurons as they become dysfunctional with old age and stress (Corona et al., 2010). Reactive microglia become hyperactive upon immune stimulation leading to increased expression of proinflammatory cytokines such as IL-1β and IL-6 that is coupled with reduced expression of anti-inflammatory cytokines, notably IL-10 (Godbout et al., 2005; Godbout and Johnson, 2009; Ye and Johnson, 2001a). Research has documented that an increase in proinflammatory and decrease in anti-inflammatory cytokine production can detrimentally affect cognition with increasing age (Barrientos et al., 2009; Chen et al., 2008; Weaver and Seeman, 2000).

Aging, Inflammation, and Microglia

In the healthy adult brain, microglia remain in a quiescent state, characterized by a small cell body, with long, fine, ramified processes that survey their environment. Microglial cells are transformed to an activated state when presented with pathogens or endogenously derived

antigenic molecules that occur during infection or following cell death/dysfunction. Microglia are also activated in response to cues from other immune cells such as proinflammatory cytokines. Microglia from aged animals express features associated with classical activation or a primed phenotype (Dilger and Johnson, 2008; Frank et al., 2006b; Perry et al., 1993; Sierra et al., 2007). Another characteristic observed in microglia from aged animals includes a molecular shift to a more phagocytic cell type as evidenced by increased expression of major histocompatibility complex molecule II (MHC II) (Frank et al., 2006a; Perry et al., 1993; Sheffield and Berman, 1998). Microglia from aged animals have increased expression in IL-1β, IL-6, tumor necrosis factor-α (TNF-α), a decrease in IL-10 and IL-4, and are more densely populated in the aged brain suggesting greater microglial proliferation (Aloisi, 2001; Frank et al., 2006a; Godbout et al., 2005; Kohman et al., 2012; Sierra et al., 2007; Ye and Johnson, 1999). Microglial priming associated with old age results in greater expression of IL-1β, IL-10, Toll-Like Receptor 2 (TLR2) and MHC II following immune challenge (i.e. LPS) (Chen et al., 2008; Godbout et al., 2005; Henry et al., 2009). This heightened brain immune response in aged animals governed by microglia is likely the key mediator for cognitive decline over the life of the animal.

2.2 Age Associated Decline in Cognitive Function

Hippocampus, Age-related Changes, and Cognitive Function

The hippocampus is a crucial structure for spatial learning and memory (Squire, 1992). Furthermore, the hippocampus is one of the specific regions in the adult brain that continues to develop new cells throughout life. This process, termed neurogenesis, refers to newly-developed cells that are produced in the subgranular zone and may be incorporated into the granule cell layer of the dentate gyrus and hippocampal circuitry (Cameron and McKay, 2001; Christie and

Cameron, 2006; Kempermann et al., 2003; van Praag et al., 2002). Neurogenesis decreases with age. New cell production in aged rats was decreased by 71-78% compared to young rats (Rao et al., 2006). Although a direct link has not been established, hippocampal neurogenesis is a potential mechanism through which aging negatively affects learning and memory. Current research is working on characterizing functional mechanisms of neurogenesis in hippocampal-dependent learning and memory processes.

Cytokines and Cognitive Decline

Some of the most prominent effects of aging are a decrease in cognitive abilities, e.g. learning and memory, that may be linked to the increased susceptibility to neurodegenerative disorders (Mattson and Magnus, 2006). Inflammation associated with immune stimulation and/or normal aging has been proposed as one of the main casual factors of cognitive decline in aged animals (Wilson et al., 2002). A wide array of evidence suggests that aging is associated with dysregulated immune and inflammatory responses resulting in an increased expression of proinflammatory mediators in the periphery such as IL-6, TNF-α, C-reactive protein (CRP) as well as in the brain; in particular, IL-6 and IL-1β (Bruunsgaard et al., 1999; Bruunsgaard and Pedersen, 2003; Chen et al., 2008; Harris et al., 1999; Ye and Johnson, 2001b).

IL-6 plays an important role in modulating cognition during normal aging. Increased circulating IL-6 is correlated with decreased cognitive test performance in old humans (mean age 75 y/o) (Weaver et al., 2002). In mice, the age-related decline in cognitive performance is attenuated in IL-6 knockout (KO) mice (Dugan et al., 2009). Deficient IL-6 expression was associated with IL-6 KO mice maintaining parvalbumin (PV) positive-interneurons (neurons essential for normal information processing, encoding, and retrieval in hippocampus and cortex)

(Dugan et al., 2009). Furthermore, mice deficient in IL-6 had increased efficiency in locating the platform during Morris water maze compared to wild type mice after LPS administration (Sparkman et al., 2006). These data suggest that high levels of IL-6 disrupt cognitive function in the aged. Another important proinflammatory cytokine, IL-1β, directly inhibits cognition and specifically hippocampal-dependent memories when present in elevated levels. However, IL-1β plays an important role in normal learning and memory as a blockade of IL-1 signaling with IL-1 receptor antagonist (IL-1RA) is detrimental to performance in water maze testing (Goshen et al., 2007; Yirmiya et al., 2002). However, elevated expression of IL-1β in the hippocampus can cause cognitive impairment and disrupt long-term potentiation (LTP), a cellular mechanism important for certain types of memory. Elevated expression of IL-1β in the hippocampus of aged mice is positively correlated with deficits in hippocampal-dependent tasks (Buchanan et al., 2008). Moreover, the level of expression of IL-1β on memory consolidation was demonstrated when peripheral administration of IL-1RA restored LPS-induced deficits (Pugh et al., 1998). Intraperitoneal administration of LPS in rats resulted in elevated IL-1β in the hippocampus, which decreased time spent frozen during the contextual fear test; however, this contextual memory impairment was recovered when rats received IL-1RA (Pugh et al., 1998). Based on these findings and numerous other reports defining the functional importance of IL-1β on learning and memory, it appears that IL-1β interferes with learning and memory if not maintained within a defined range (Yirmiya and Goshen, 2011). As animals age, IL-1β levels slowly increase in brain, and this increase may play a causative role in the decreased cognitive performance and heightened neuroinflammation observed in old animals. Taken together, these studies demonstrate the importance of increased levels of proinflammatory cytokine expression in the aged brain and how they can impact age-related cognitive decline.

Neurotrophic Factors and Cognition

Aging is associated with a decline in neurotrophic factors such as BDNF, IGF-1, and vascular endothelial growth factor (VEGF) that support neuronal health. A decline in neurotrophins contributes to the age-associated decrease in cell proliferation (Erickson et al., 2010; Shetty et al., 2005; Silhol et al., 2005; Sonntag et al., 2005). IGF-1 impacts neuronal functions that are important for learning and memory (Sonntag et al., 2005). Administration of exogenous IGF-1 reverses cognitive aging in rodents. Working memory was increased in aged rats during Morris water maze testing compared to aged-matched control rats after chronic intracerebroventricular injection (i.c.v.) infusion of IGF-1 (Markowska et al., 1998). BDNF is another highly expressed growth factor that promotes survival, differentiation, and neuronal support to the hippocampus (Ernfors et al., 1990; Maisonpierre et al., 1990; Thoenen, 1995). Aged animals have reduced BDNF compared to young animals, leading to increased vulnerability of neurons to damage in aged animals and potential impairment in learning and memory. BDNF plays a pivotal role in hippocampal-dependent learning. Young adult BDNF knockout (KO) mice displayed significant spatial learning impairment in the Morris water maze, requiring twice the number of days to master the task compared to young and aged wild-type mice; additionally, aged BDNF KO mice were unable to learn the task (Linnarsson et al., 1997). In humans, BDNF levels are reduced in patients suffering from AD (Hock et al., 2000; Phillips et al., 1991). Thus, a decrease in BDNF may contribute to alterations that underlie age-related cognitive decline and increased susceptibility to neurodegenerative disorders.

VEGF is another important growth factor with regard to maintaining a healthy brain. As observed with IGF-1 and BDNF, VEGF is also significantly reduced in middle and old aged animals, with VEGF expression declining to half of the levels observed in young adults (Shetty

et al., 2005). VEGF is most notable in promoting angiogenesis; however, VEGF also positively contributes to learning and memory. Adult rats overexpressing VEGF in the hippocampus display reduced latency and pathlength during Morris water maze testing and enhanced spatial memory during probe testing (Cao et al., 2004). Environmental enrichment increases expression of VEGF and results in greater neurogenesis that is associated with improved cognition; however, inhibition of environmental induction of VEGF by RNAi completely blocks neurogenesis (Cao et al., 2004). In conclusion, there is strong support for a beneficial role of neurotrophins in hippocampal-dependent learning and memory, and decreased neurotrophin expression likely contributes to cognitive impairment in the aged.

2.3 Interventions for Suppressing Age Associated Heightened Inflammation and Decline in Cognition

Physical activity and EGCG dietary supplementation have been shown to affect several parameters of age-related cognitive decline under various experimental conditions (Assuncao et al., 2011; Gomes da Silva et al., 2013; Jia et al., 2013; Kohman et al., 2012; Kramer et al., 2006; van Praag et al., 2005; Wang et al., 2012). Additionally, β-ala is purported to have anti-oxidant properties and is proposed to act as an anti-aging therapeutic (Artioli et al., 2010; Hipkiss, 2009). The dietary intervention is expected to act directly (EGCG) within the brain through different anti-oxidant and anti-inflammatory properties (Kim et al., 2007; Mandel et al., 2005; Sutherland et al., 2006) and indirectly (β-ala) by improving exercise capacity (Artioli et al., 2010; del Favero et al., 2012). Exercise has proven to be an effective intervention for improving age-related cognitive decline, thus the combination of diet and exercise suggests that their effects on the

brain can be complimentary and enhance the overall effect on hippocampal molecular measures and cognitive performance.

Exercise

Exercise is frequently used as an intervention to improve cardiovascular health; however, aerobic exercise is becoming increasingly important for maintaining and possibly enhancing brain health. Aerobic exercise improves brain function in older adults (Colcombe et al., 2004; Erickson et al., 2011; Kramer et al., 2006; Voss et al., 2010) and slows the progression of neurodegenerative disorders such as PD (Archer et al., 2011) and AD (Archer, 2011). Additionally, rodent studies consistently show improved performance on cognitive tasks, particularly hippocampus-dependent tasks (i.e. Morris water maze and contextual fear conditioning), when rodents engage in voluntary wheel running (Baruch et al., 2004; Clark et al., 2008; van Praag et al., 2005; Vaynman et al., 2004).

Exercise induces a host of changes within the CNS and contributes to the integrity of the hippocampus. Exercise increases the production of neurotrophins promoting growth and repair, vascularization, long-term potentiation, and synaptic plasticity (Berchtold et al., 2010; Christie et al., 2008; Clark et al., 2008; Farmer et al., 2004; Greenwood et al., 2009; Rhyu et al., 2010). In addition, exercise enhances cell proliferation in the hippocampus, a process that may improve cognition (Lemaire et al., 1999; Shors et al., 2001; van Praag et al., 1999a; van Praag et al., 1999b). Unlimited access to running wheels for one month improved learning and memory in old rats to levels seen in young rats (van Praag et al., 2005).

Many different molecular and cellular pathways may mediate the effects of exercise on cognition and behavior. The production of growth factors and cell proliferation, which are

induced with chronic aerobic exercise, are important to learning and memory. Benefits of exercise are still evident in aged animals and may be an important life style intervention for maintaining brain health throughout the life-span. Exercise reliably leads to an increase in cell proliferation in the dentate gyrus (DG) of aged animals (Creer et al., 2010; van Praag et al., 2005; Vivar et al., 2012). This proliferation of cells coupled with increased survival of neurons in the hippocampus may be the key mediator that leads to improved learning (Clark et al., 2008; Creer et al., 2010)**.** However**,** the effects of exercise are complex and thus many other factors could contribute to its benefits on cognition and brain health.

Exercise also has numerous effects on immune function in the periphery and this leads to improved health of the peripheral nervous system (Woods et al., 2009). The modulatory effects of exercise to skew microglia to an alternative activation/anti-inflammatory phenotype are gaining attention. Microglia are implicated in the low-grade chronic inflammation observed in the aged brain and current literature suggests that exercise may have direct effects on microglial cell activity (Kohman et al., 2012). Treadmill running attenuated the aged-dependent increase in microglia activation in a transgenic mouse model of AD (Nichol et al., 2008). Wheel running offered some protection against the negative effects of immune activation (*E. coli* or LPS) and attenuated microglia cytokine production by reducing IL-1β and TNF-α (Barrientos et al., 2011). One possibility for how exercise suppresses microglia activity is via Fractalkine:Fractalkine Receptor signaling between neurons and microglia, respectively. Neurons direct microglia to be less immune active and more supportive of neuronal health by expressing fractalkine to bind to fractalkine receptors on microglia (Cardona et al., 2006). Neural precursor cell (NPC) activity within the hippocampus is influenced by microglia signaling through fractalkine signaling (Vukovic et al., 2012). Aged mice have reduced levels of fractalkine. Aerobic exercise

effectively restored fractalkine levels in aged mice to levels similar to younger animals and lead to improved NPC activity and neurogenesis (Vukovic et al., 2012). Additionally, wheel running significantly increased the proportion of Iba-1 positive cells that co-expressed IGF-1 in both aged and adult mice, signifying a possible explanation in reducing central inflammation and enhancing hippocampal neurogenesis (Kohman et al., 2012). Given the involvement of the hippocampus in learning and memory, changes in the microenvironment of microglial cells may be of particular relevance to cognitive function.

Another beneficial effect of exercise on microglia in the aged brain is that exercise may improve the balance of proinflammatory and anti-inflammatory cytokines. Exercise increased the expression of the anti-inflammatory cytokine IL-10 in aged rats and although exercise did not decrease proinflammatory cytokines (IL-1β, IL-6, TNF-α) in this study, there was a significant reduction in the ratio of pro- and anti-inflammatory cytokines detected compared to the non-exercised group (Gomes da Silva et al., 2013). Exercise may also be beneficial for regulating cross-talk between immune function and neurotrophic support function of microglia. This interaction between cytokines and growth factors is important as exercise-induced increases in growth factors may help protect against the negative effects of increased proinflammatory cytokine expression. After 6 weeks, running improved hippocampal-dependent memory and increased BDNF mRNA in the dentate gyrus (Greenwood et al., 2009). Similarly, exercise caused an upregulation of BDNF expression as well as an increase in adult hippocampal-neurogenesis (Kobilo et al., 2011). BDNF is considered the most important factor upregulated by physical activity and BDNF can be downregulated by IL-1β (Barrientos et al., 2004; Cotman and Berchtold, 2007). Therefore, the importance of running in reducing the sensitivity of microglia to age-related increases of IL-1β may be a core mechanism by which running protects aged

subjects. The upregulation of neurotrophins (BDNF, IGF-1, and VEGF) associated with exercise may help offset age-related reductions in synaptogenesis, neurogenesis, angiogenesis, and learning and memory. In conclusion exercise is a powerful lifestyle intervention that could be used to augment and maintain cognitive function throughout the lifespan.

Green Tea and Health Benefits

The largely consumed polyphenol-rich beverage, green tea, has attracted worldwide attention. Endowed with biological and pharmacological activities, tea is considered a dietary source of bio-active constituents with potential health benefits. The beneficial effect of green tea is thought to be due to its high content of polyphenolic flavonoids.

Catechin polyphenols modulate the cellular redox state by acting as radical scavengers of oxygen and nitrogen species (Mandel et al., 2005). These polyphenols are also known to have therapeutic health effects for a variety of chronic pathological conditions including cancer, neurodegeneration, diabetes, and cardiovascular disease (Corcoran et al., 2012; Kishimoto et al., 2013; Mandel et al., 2008). Apart from the accepted multiple biological anti-oxidant properties and anti-apoptosis, green tea polyphenols show neuroprotective effects in animal models of PD, AD, and ischemic stroke (Mandel and Youdim, 2004; Pan et al., 2003).

In animals, tea catechins increased adult neurogenesis presumably by reducing inflammation (Li et al., 2004; van Praag et al., 2007; Wang et al., 2012; Yoo et al., 2010). Long term oral administration of green tea catechins in drinking water prevented age-related spatial learning and memory decline in mice by affecting hippocampal CREB signaling (Li et al., 2009). Furthermore, green tea polyphenols can modulate the proinflammatory cytokines such as TNF-

α; as well as, inducible nitric oxide synthase in peripheral macrophages (Calixto et al., 2004; Yang et al., 1998).

Tea consumption has been inversely correlated with incidence of dementia, AD, and PD (Checkoway et al., 2002; Hu et al., 2007). Epidemiological findings show that consumption of green tea is positively associated with lower risk of PD (Ascherio et al., 2001; Chen et al., 1998). One study found higher consumption of green tea (>2 cups/day) was associated with lower prevalence of cognitive impairment in humans (Kuriyama et al., 2006). A case-control study in the United States and a prospective cohort study in Finland found that people who consumed 2 or more cups/day of green tea presented a decreased risk for PD (Checkoway et al., 2002; Hu et al., 2007). Thus tea drinking may protect the brain as we age. Together these studies show that green tea has beneficial effects by acting as a neuroprotectant and decreasing age-related cognitive decline.

EGCG

Fresh green tea leaves are particularly rich in catechins and HPLC analysis of green tea shows that epigallocatechin-3-gallate (EGCG) is the most abundant biologically active component, accounting for over 60% of the catechins (Ramassamy, 2006). The 3',4'-dihydroxyl group in the B ring as well as the gallate group are structurally important features that define the catechins chelating potential and protecting cells against oxidative damage (Grinberg et al., 1997; Guo et al., 1996; Hider et al., 2001). EGCG is absorbed into the enterocytes and then undergoes glucuronidation, sulfation, or methylation in the liver (Manach and Donovan, 2004). EGCG readily undergoes the phase II biotransformation reaction of methylation to form 4"-*O*-methyl-(-)-EGCG and 4',4"-*O*-dimethyl-(-)-EGCG from catechol-*O*-methyltransferase (Lu et al.,

2003). Absorption takes place in the enterocytes and substantial quantities pass from the small to the large intestine where EGCG is further degraded by the microbiota; therefore, it is suggested that not much EGCG gets into the blood (Auger et al., 2008; Lee et al., 2002; Roowi et al., 2010; Stalmach et al., 2010). Peak plasma concentration of EGCG in healthy subjects with one oral dose in the morning after an overnight fast was reached in 1-2 h. After 24 hours the levels of EGCG were undetectable with the elimination half-life reached at 3.4 ± 0.3 h (Lee et al., 2002). Overall, EGCG can be absorbed after oral administration and has been shown to exert biological activities in vivo.

The biological activity of EGCG is considered to be associated with its antioxidant potential and metal-chelating properties (Mandel and Youdim, 2004; Mandel et al., 2005). Several studies have demonstrated the antioxidant activity of EGCG. Inhibition of reactive oxygen species (ROS) resulted from EGCG's ability to significantly inhibit the enzyme xanthine oxidase (Aucamp et al., 1997). In addition, EGCG attenuated LPS-induced inducible oxide synthase (iNOS) expression by 40-50% in peritoneal macrophages (Chan et al., 1997; Lin and Lin, 1997). EGCG inhibits iNOS and prostaglandin-endoperoxide synthase 2 (COX-2) expressions from an increased inflammatory response related to LPS in brain and cultured astrocytes (Lee et al., 2013). However, data indicate that EGCG may likely function under a wide spectrum of mechanisms of action contributing towards the "neuroprotective" and "neurorescue" capacities beyond just antioxidant features (Mandel and Youdim, 2004; Weinreb et al., 2004).

EGCG has been shown to protect against brain inflammation and improve age-related cognitive decline and neurogenesis (Kim et al., 2007; Wang et al., 2012; Yoo et al., 2010).

Gavage administration displayed anti-inflammatory properties of EGCG by downregulating NFκB and displaying a ROS-blocking capacity on neurons, reducing brain inflammation and neuronal damage in an experimental autoimmune encephalomyelitis (EAE) model (Aktas et al., 2004). Furthermore, EGCG has been found to be a potent inhibitor of microglial activation. An *in vitro* study demonstrated that EGCG inhibits LPS-activated microglial secretion of nitric oxide (NO) and TNF-α through the down regulation of iNOS and TNF-α gene expression (Li et al., 2004). Therefore, it is reasonable to speculate that EGCG may act as a therapeutic agent to alleviate microglia activation involved in neurodegeneration.

Dietary EGCG is known to have beneficial effects on age-related cognitive decline. For example, a 4 week EGCG treatment by gavage rescued spatial learning and memory in 30 mo female APP/PSI mice (Jia et al., 2013). Treatment with EGCG in drinking water for 1 week improved learning and memory in Morris water maze (Lee et al., 2009). In connection to cognition, EGCG was observed to increase cell proliferation and increase the number of neuroblasts in the DG (Yoo et al., 2010). This is supported by enhanced adult hippocampal-neurogenesis by acting directly on adult hippocampal NPCs favoring neuron production (Wang et al., 2012). Taken together, accumulating evidence suggests that EGCG has potential to not only protect the aged-brain from inflammation and oxidative stress but to act as a neuroprotectant and improve age-related cognitive decline.

β-alanine

β-alanine (β-ala) is a nonessential (endogenously produced) non-proteogenic amino acid that is synthesized in the liver (Matthews and Traut, 1987). Dietary β-ala is mainly found in

meats (i.e. beef, chicken, and turkey) and is absorbed into skeletal tissue for synthesis of carnosine (Hoffman et al., 2012).

A popular area of study focuses on β-ala and physical activity. β-ala supplementation increases muscle carnosine, which improves muscle function and decreases fatigue during high intensity exercise (Artioli et al., 2010). Additionally, β-ala's potential effects in the brain have garnered recent attention. β-ala occurs naturally in the CNS, acts as a structural intermediate between amino acid neurotransmitters glycine and γ-aminobutyric acid (GABA), and is recognized by multiple receptors in the CNS (Tiedje et al., 2010). However, limited research has examined the effects of β-ala in the brain, and it should be noted that its exact role in the CNS is not presently clear. A recent study examined whether β-ala levels in brain tissues of rats were associated with improved test performance in Morris water maze. Interestingly, β-ala was increased in the hippocampus of tested rats 5 minutes and 6 hours after a Morris water maze probe trial, indicating a direct role as a neurotransmitter or indirectly through carnosine (Sase et al., 2013). Therefore, the authors speculated that β-ala may be involved in retrieval of spatial memory.

β-ala supplemented for 12 weeks in elderly men and women (60-80 y/o) resulted in significant increase in muscle carnosine (del Favero et al., 2012). It is thought that increased carnosine may be a mechanistic component of β-ala's benefits. Carnosine is a cytoplasmic dipeptide composed of amino acids β-ala and histidine (Harris et al., 1990). The physiological role of carnosine is maintenance of acid-base homeostasis (Harris et al., 1990). Although carnosine is mainly concentrated in skeletal muscle, it is also expressed in heart and in brain, particularly in glia and neuronal cells (Boldyrev et al., 2010; del Favero et al., 2012; Quinn et al.,

1992; Stout et al., 2007). Because carnosine absorption from circulation is limited, it requires endogenous synthesis within the skeletal muscle (Skaper et al., 1973). β-alanine is the rate-limiting peptide in carnosine synthesis, and supplementation with β-ala increases carnosine synthesis within the skeletal muscle (Derave et al., 2007; Harris et al., 2006; Hill et al., 2007). Carnosine is an antioxidant, scavenging ROS, aldehydes, ketones, and inflammatory cells (Boldyrev, 2005). Deficiency of muscle carnosine may increase oxidative stress-induced lipid and protein oxidation and increase inflammation (Bellia et al., 2011). Interestingly, decreased muscle and plasma carnosine concentrations have been reported in patients with AD compared to age-matched controls (Fonteh et al., 2007). It is possible that declining levels of carnosine contribute to elevated oxidative stress in AD patients. The biological role of carnosine as an antioxidant encourages further examination of its potential involvement in pathology of AD and other disorders associated with heightened oxidative stress (Hipkiss, 2007).

To date, limited research has examined whether β-ala works directly or indirectly through carnosine, and its potential ability to improve both skeletal muscle function and cognition in aged models is not well-defined. Further research is necessary to determine if dietary supplementation with β-ala is an effective approach to combat age-related oxidative stress in brain and muscle.

2.4 Conclusion

Age-related cognitive decline is a growing concern with substantial personal, social, and economic costs and has led to calls for interventions to prevent or slow this devastating disorder in the rapidly increasing aged population. Individually, research suggests physical exercise, EGCG, and β-ala may show promise for combating losses in cognition due to aging. However,

research examining the additive effects of combining these interventions has not been investigated. Therefore, the examination of the potential for exercise and dietary supplementation to have an additive effect to improve learning and memory in normal aged mice will be beneficial to the field of aging research with potential application as a nutraceutical therapy for aged humans.

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Chapter 3

Voluntary wheel running, but not a diet containing (-)-epigallocatechin-3-gallate and β-alanine, improves learning, memory and hippocampal cell proliferation in aged mice.

3.1 Introduction

The percentage of the population 60 years of age and older is rapidly growing worldwide (United Nations 2012) and therefore, research investigating therapeutic interventions to combat aging-related pathological changes in the brain has garnered high interest (Abraham and Johnson, 2009; Jang et al., 2010; and Woods et al., 2012). Deterioration of brain function is a consequence of normal aging leading to cognitive impairments that are independent of disease (Barrientos et al., 2010; Wynne et al., 2009; and Perry et al., 1993). In particular, the functional integrity of the hippocampus is vulnerable to the aging process impacting learning and memory in various animal models and in humans (Amrein et al., 2011; Kuhn et al., 1996; Lucassen et al., 2010; and Porte et al., 2008). During aging, pathological changes in the brain include increased production of pro-inflammatory cytokines and higher levels of oxidative stress that, in turn, can precipitate a decrease in adult hippocampal neurogenesis and decline in learning and memory. Down regulation of neurotrophic factors, most notably brain-derived neurotrophic factor (BDNF), has been linked to cognitive decline and the age-related decrease in neurogenesis (Barnes and Thomas, 2008; Sanchez et al., 2011; Schulte-Herbruggen et al., 2008; Silhol et al., 2005; and Ziegenhorn et al., 2007). BDNF plays a critical role in synaptic plasticity, learning and memory (Poo, 2001) and promotes hippocampal neuron survival and differentiation (Cheng and Mattson, 1994; Lee et al., 2002; and Lindholm et al., 1996). The complex interaction of increased pro-inflammatory cytokines, increased oxidative stress, decreased neurotrophin

expression and decreased neurogenesis promotes a microenvironment conducive to age-related cognitive decline.

Green tea, a polyphenol-rich beverage has drawn much attention due to its health benefits in cardiovascular disease, diabetes, inflammatory diseases, and its prevention and treatment of cancer (Schneider and Segre, 2009). Green tea is rich in flavonoids and contains many catechins, including (-)-epigallocatechin-3-gallate (EGCG), which is the most abundant (~60%) catechin in green tea (Cabrera et al., 2006). Green tea consumption is related to lower prevalence of cognitive impairment in aged humans (Kuriyama et al., 2006) and, in animals, tea catechins increase adult neurogenesis perhaps by reducing neuroinflammation (Li et al., 2004; van Praag et al., 2007; Wang et al., 2012; and Yoo et al., 2010). EGCG reduces microglia activation (Li et al., 2004; Wu et al., 2012), oxidative stress (Mandel et al., 2005, Sutherland et al., 2006), and inflammation (Kim et al., 2007). Based on these observations, catechins from green tea are thought to act as a general neuroprotective factor helping to prevent neurodegenerative diseases (Mandel et al., 2005).

β-alanine (β-ala) is the β form of the amino acid alanine and is a precursor molecule for carnosine (Tiedje et al., 2010). β-ala supplementation and subsequent increases in muscle carnosine have been shown to improve muscle function and decrease fatigue in high intensity exercise (Artioli et al., 2010). β-ala is used as a dietary supplement by athletes for this reason. Less is known about the effect of β-ala on learning or memory. A recent study demonstrated an increase in β-ala in the hippocampus of rats at 5 minutes and 6 hours after a Morris water maze probe trial (Sase et al., 2013), a finding the authors speculate may indicate a role for β-ala in retrieval of spatial memory. β-ala is thought to act as a neurotransmitter in the hippocampus, is a structural intermediate between established amino acid neurotransmitters glycine and γ-

aminobutyric acid (GABA), and is recognized by multiple receptors in the central nervous system (CNS) (Tiedje et al., 2010). However, the exact role of β-ala in the CNS is not presently clear. L-carnosine, a dipeptide of β-ala and L-histidine, has been studied for its effects on cognition. L-carnosine supplementation improved cognitive flexibility and efficiency as well as reaction time in schizophrenic patients (Chengappa et al., 2012). It is hypothesized that the CNS effects of L-carnosine supplementation are mediated, at least in part, by its potent anti-oxidant effects (Bolyrev et al., 1997; Hipkiss et al., 1998); a theory supported by the protective role of carnosine against cerebral ischemia in animal models (Dobrota et al., 2005; Rajanikant et al., 2007). However, as with β-ala, the functional role of L-carnosine in the CNS remains poorly defined.

In contrast, a large body of literature has established that regular exercise increases adult neurogenesis in the hippocampus of mice, and this increase has been related to improved spatial memory in the Morris water maze (van Praag et al., 1999; van Praag et al., 1999a), radial arm maze (Anderson, 2000), and y-maze (van der Borght, 2007). Our group has shown that adult hippocampal neurogenesis is required for exercise-induced improvements in spatial memory, as irradiation-induced reduction in neurogenesis was sufficient to eliminate the positive wheel running effect on performance in the Morris water maze (Clark et al., 2008). BDNF is thought to play a major role in the increase in neurogenesis and learning/memory as a result of physical exercise (Barde et al., 1994; Lo, 1995). Inhibition of the exercise-induced increase in BDNF action in mice abrogates the exercise-related improvements in spatial memory (Vaynman et al., 2004). Importantly, our recent study in older adults demonstrated an exercise training-induced increase in circulating BDNF concomitant with increases in hippocampal volume and performance on memory tasks (Erickson et al., 2011).

Despite the number of studies that have demonstrated that exercise and dietary supplementation with EGCG or β-ala are beneficial for preventing or recovering age-related deficits individually, no research has been conducted that investigates whether additive or synergistic effects between dietary supplementation with EGCG, β-ala and exercise exist for enhancing age-related cognitive loss and reducing oxidative stress and inflammation in the aged brain. Moreover, most studies have utilized long-term (e.g. 6-7 months) administration (Assuncao et al., 2010; Assuncao et al., 2011; Li et al., 2009; Li et al., 2010; and Rodrigues et al., 2013) of catechins as a means of *preventing* age-related cognitive loss. It is unclear whether cognitive loss and age-related hippocampal changes can be *reversed* with short-term supplementation with or without exercise. We hypothesized that exercise supplemented with dietary EGCG and β-ala would reduce neuroinflammation, increase the formation of new cells in the dentate gyrus of the hippocampus and improve performance in tests of spatial learning and memory when compared to each treatment (e.g. exercise or EGCG/β-ala) individually and that all interventions would significantly improve all outcomes relative to untreated aged mice.

3.2 Materials and Methods

Animals. For all studies, aged (19 month old) male BALB/cByJ mice were utilized. Retired breeder mice (8-10 months old) were purchased from Jackson Labs (Bar Harbor, ME) and aged in our facility. Upon arrival at our facility, all mice were fed 8640 Teklad 22/5 rodent diet (Harlan Teklad, Indianapolis, IN) and autoclaved water ad libitum. Mice were individually housed in polypropylene cages under a reverse 12-h-light/-dark cycle at 24°C. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Diets containing EGCG and β-ala. At the start of the intervention (e.g. 18-19 mos. old), mice received either a control diet (AIN-93M, Research Diets, New Brunswick, NJ) or an experimental diet containing 1.5 mg Teavigo (90% EGCG, DSM Nutritional Products, Basel, Switzerland) and 3.43 mg β-ala (NutraBio, Middlesex, NJ) per g of AIN-93M diet. The experimental diet was mixed by Research Diets (New Brunswick, NJ). A sample of each diet was assayed independently by Covance, Inc. (Princeton, NJ), and found to contain 99.3% and 97.4% of expected EGCG and β-ala contents, respectively. The compounds were stable in the diet for at least 4 months. The control diet was found to be free of both EGCG and β-ala. Dietary components are listed in **Table 3.1**. Based upon the amount of diet disappearance and mouse body weights, we estimated that mice ingested on average 182 mg/kg/day and 417 mg/kg/day, of EGCG and β-ala, respectively. The rationale for the EGCG dosage was based on previous studies demonstrating beneficial effects of EGCG on cognition in mice (Li et al., 2009; Li et al., 2010). As there are few studies examining the effects of β-ala supplementation on cognition or muscle function in mice, our β-ala dosage was calculated from the effective dose in aged humans of 2.4 g/d that led to improved physical work capacity (Stout et al., 2008). For a 70 kg person, this would equate to 34 mg/kg/d. The dose was adjusted for species using the FDA-recommended conversion factor of 12.3 (Food and Drug Administration, 2005) resulting in a target dose of 418 mg/kg/d.

Voluntary Wheel Running (VWR). VWR mice were housed in cages with access to a running wheel (Respironics, Bend, OR). Sedentary (SED) mice were housed without a running wheel in standard shoebox cages. Wheel distance was continuously monitored and recorded every hour by a computerized system (VitalView software, Respironics, Bend, OR) and analyzed as wheel distance per 24 hour period. Mice remained in wheel or shoebox cages throughout the duration

of the study, including during the behavioral testing period. Mice were euthanized prior to the onset of the dark cycle on the final day of the study, thus they had remained sedentary (i.e. without significant wheel activity) for ~11 hours prior to tissue collection to control for any acute effects of wheel running.

Study Design. Mice were provided AIN-93M diet or the experimental diet containing EGCG and β-ala for 28 days prior to and during an 11 day test period for spatial learning and memory (**Figure 3.1**). Food and water disappearance and body weight were measured twice-weekly during the study. Bromodeoxyuridine (BrdU) was injected at a dose of 50 mg/kg i.p. for 10 consecutive days at the start of the experiment to label proliferating cells. After the 28 days, the Morris water maze was used to determine hippocampal-dependent learning and memory over a 7 day period. After a two-day reprieve, mice underwent the hippocampal-dependent contextual fear conditioning task (**Figure 3.1**). All behavior testing occurred during the dark phase of the light/dark cycle. Animals were euthanized by CO_2 asphyxiation 24 hours after the final test.

Morris Water Maze (MWM). Mice were handled daily for 7 days prior to assessment of learning and memory to habituate them to being manipulated during the testing period. In this test the animals use distinctive visuospatial cues surrounding the pool to navigate a direct path to the hidden platform (D'Hooge and De Deyn, 2001; Jurgens et al 2012; and Morris, 1984). A circular pool (100 cm diameter, 23-26°C) with a round platform (10 cm diameter) hidden 0.5 cm below the surface of opaque water was positioned in one of the four quadrants. The platform remained in the same quadrant during the 5 day acquisition testing. Trials were conducted using a pseudorandom protocol in which mice were placed in the water in one of the three quadrants not containing the platform. Animals were placed in the water and allowed to swim freely for 60 s or until the platform was reached. If mice did not locate the platform in 60 s, they were guided to

the platform and allowed to remain on it for 20 s. Animals were removed from the pool and returned to their home cage for 10 s before repeating the trial. After completion of four consecutive trials, mice were placed in their home cage under a heat lamp until dry. On day 5, the platform was removed and mice received a 60 s probe trial to assess memory for the platform location. On the sixth day, mice were subjected to two days of reversal testing in which the hidden platform was moved to the opposite quadrant of the pool, but all the distal visual cues remained constant. Reversal learning measures how quickly an animal is able to extinguish memory of the initial position of the platform and learn the new location. One hour after the last mouse completed reversal testing, the platform was removed and mice received a 60 s reversal probe trial to assess learning of the platforms new location. A video camera mounted to the ceiling directly above the center of the maze was used in conjunction with a computerized animal tracking system (EthoVision; Noldus Information Technologies, Netherlands) to calculate swim speed (cm/s), latency to platform (s), and distance swam (pathlength to platform) (cm).

Contextual Fear Conditioning (CFC). Mice were individually placed into a square chamber (32 cm L x 28 cm W x 30 cm H, dark grey walls) with a metal bar grid floor connected to a shock scrambler controlled by a digital timer (Med Associates, St. Albans, VT, USA). On the first day, mice were placed into the chamber and allowed to acclimate for two minutes. After the acclimation period, the mice were presented with a tone for 20 seconds. During the last two seconds of the tone, a 0.75 mA shock was delivered to the feet. The same tone and shock delivery pattern was repeated 60 seconds later (at 200 seconds). The mouse remained in the chamber for an additional 30 seconds before being returned to their home cage. On the second day, mice were tested for freezing (immobility) in either the original or novel contexts. The novel context was an octagon shaped chamber with white and black striped walls and a smooth

floor. The presentation of the contexts was counterbalanced with half of the mice being first placed in the novel context and then the original context or vice versa. When placed in the original context, square box with the grid floor, mice remained in the chamber for a total of 250 seconds in absence of tone or shock. When placed in the novel context for 250 seconds, mice were presented a tone at 120 and 200 seconds. There was no shock delivered in either context during the testing phase. Freezing and distance traveled was recorded by TopScan video tracking software (CleverSystems, Reston, VA, USA). Freezing is the total number of seconds when the animal's center of mass did not register horizontal movement ($\leq$ 1 mm). Freezing data were converted into percent time spent freezing by dividing the total number of seconds a mouse spent freezing by the total number of seconds of testing (250 seconds) multiplied by 100. Dependent variables are distance traveled (cm) and percent time frozen.

Tissue Collection. Mice were euthanized by CO_2 asphyxiation then intra-cardially perfused with saline. Brains were removed and longitudinally cut into hemispheric sections. One half of the brain was dedicated to immunohistochemistry and was immediately placed in 4% paraformaldehyde to fix overnight. The other half was dedicated to dissection of brain regions (hippocampus and cerebellum) before snap frozen in liquid nitrogen and stored at -80°C until processing.

Immunohistochemistry. After overnight fixation in 4% paraformaldehyde, the tissue was transferred into 30% sucrose solution. Brains were sectioned at 40 µm using a cryostat. A one-in-six series was stained for bromodeoxyuridine (BrdU) to identify newly divided cells. Immunohistochemistry was performed according to Kohman et al. (2012) using rat anti-BrdU (1:100; AbD Serotec, Raleigh, NC) as the primary antibody and biotinylated goat anti-rat (1:250; Vector Laboratories, Burlingame, CA) as the secondary antibody. After incubation in the

secondary antibody, sections were then treated with the avidin/biotinylated enzyme complex ABC system (Vector, Burlingame, CA) and stained using a diaminobenzidine kit (DAB; Sigma, St. Louis, MO).

BrdU$^+$ positive cell identification. Estimates of total number of BrdU$^+$ positive cell counts have been previously described (Kohman et al, 2012). Briefly, the entire granule layer (bilateral) of the dentate gyrus was outlined, and BrdU$^+$ positive nuclei were automatically counted by a validated fixed threshold to remove the background for each image. The fraction of cells predicted to cross the boundary were removed to produce unbiased estimates.

Hippocampal Gene Expression. Total RNA from homogenized hippocampal tissue was isolated using the Tri Reagent protocol (Sigma, St. Louis, MO, USA). Synthesis of cDNA was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). A custom Taqman® Low Density Array (TLDA) card (Applied Biosystems, Foster City, CA, USA) was designed for use with hippocampal cDNA, which contained reference genes (18S rRNA and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) and genes of interest (**Table 3.2**). Plates were loaded with cDNA, converted from 1000 ng of total RNA, mixed with an equal volume of Taqman® Universal PCR Master Mix (2x). Amplification was run according to the manufacturer's instructions on a Prism 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data analysis was conducted using RQ Manager (Applied Biosystems, Foster City, CA, USA). Data was analyzed using the comparative threshold cycle (Ct) method (Livak KJ and Schmittgen TD, 2001) and results are expressed as relative change fold to the standardized relative quantification (RQ) baseline (RQ =1). Control-sedentary (Con-SED) was set as the calibrator/control group. A value higher than "1" would represent a fold

increase in mRNA expression relative to control for a particular gene, while a value between "0" and "1" would represent a fold decrease in mRNA expression relative to control.

Oxidative Stress. 4-hydroxynonenal (4-HNE) was analyzed in cerebellar extracts by enzyme-linked immunosorbant assay (OxiSelect HNE Adduct ELISA Kit, Cell Biolabs, San Diego, CA) according to manufacturer's instructions. Briefly, cerebellums were homogenized in sterile PBS supplemented with 5 µl of protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO). Samples were assayed for protein content by DC Protein Assay (Bio-Rad, Hercules, CA) and diluted to 10 µg total protein·ml^{-1} in PBS for use in the kit then immediately used for 4-HNE assays. All samples were run in duplicate.

Statistics. Descriptive (**Table 3.3**) and MWM acquisition data were analyzed by 2-way (exercise x diet) ANOVA with repeated-measures. CFC, hippocampal gene expression, neurogenesis and 4-HNE data were analyzed by ANOVA using a 2 x 2 arrangement of treatments. MWM probe and reversal trials were analyzed using chi square analyses. Appropriate post-hoc analyses were used in the event of a significant interaction or main effect of exercise or diet. Significance was set at $p < 0.05$. Statistical analyses were performed using SPSS software version 22 (IBM Corp., Armonk, NY). All values are means ± SEM with an n=8-15/treatment combination dependent on the variable.

3.3 Results

Descriptive Data. At the initiation of the diet and exercise intervention, body weight between treatment groups was not different (**Table 3.3**). As expected, VWR resulted in a significant ($F_{1,52}$=37; $p<0.001$ for time x VWR) reduction in body weight. There was no effect of the diet on body weight over time. Food disappearance was not significantly different between groups.

Water disappearance was significantly ($F_{1,52}$=6.0; p=0.02) increased (~15%) in response to consumption of a diet containing EGCG and β-ala when compared to the control diet. There were no significant treatment-induced differences in spleen weight. Mice in the VWR group ran ~4.6 km/day and there were no differences between those on control or EGCG/β-ala supplemented diets (**Table 3.3**).

Morris Water Maze. Analysis of pathlength to the hidden platform revealed that all of the mice acquired the task as pathlength decreased across the five days of testing ($F_{4,208}$ = 34.3, p<0.001; **Figure 3.2a**). There was a significant VWR x day interaction ($F_{4,208}$ = 3.0, p=0.02) in latency (e.g. time to reach hidden platform) as VWR mice found the platform quicker on days 3-5 **(Figure 3.2b**). The decrease in latency over the 5 day period corresponded to significantly ($F_{4,208}$ = 5.6, p<0.001, for time x VWR) faster swim speeds in the VWR mice at Days 3-5 of testing (**Figure 3.2c**). There was no effect of VWR on path length, and no effect of any experimental diet on path length, latency or swim speed relative to sedentary mice on the control diet.

On Day 5 of training, the platform was removed for a 60 s probe test 1 h after the last trial to test recall. Analysis of time spent in the target zone did not reveal any significant main effects (data not shown). However, the number of platform location crossings was greater ($F_{1,52}$ = 9.3, p=0.004) in the VWR mice (**Figure 3.3a**).

To assess spatial working memory, the hidden platform was moved to the opposite quadrant for two days of reversal training (e.g. Days 6 & 7, **Figure 3.1**). Animals must be able to incorporate new information (i.e. new platform location) with existing memories (spatial locations of visual cues) in order to complete this task. Two days of reversal training did not

reveal any significant main effects for diet or VWR (or their interaction) in path length or latency, despite faster swim speeds for VWR (data not shown).

A 60 s probe test was performed on Day 7 after two days of reversal testing 1 h after the last reversal test. As was the case for the Day 5 probe test, we found a significant ($F_{1,52}$ = 5.1, p=0.03) main effect for VWR for the number of platform location crossings (**Figure 3.3b**). The experimental diet containing EGCG and β-ala was without effect.

Contextual Fear Conditioning. As expected, animals subjected to shock during training (day 38) demonstrated a significant ($F_{1,48}$=16.8, p<0.001) increase in freezing time during the subsequent (day 39) exposure to the original context compared to those that did not receive a shock (data not shown). Analysis of treatment differences in shocked mice revealed that mice in the VWR groups exhibited significantly ($F_{1,48}$=8.1, p=0.007) elevated freezing behavior (**Figure 3.4a**). There was no effect of diet. In the novel context test on Day 39, VWR mice displayed a significantly ($F_{1,38}$=4.9, p=0.03) elevated freezing behavior in the absence of a tone (**Figure 3.4b**). Freezing behavior was elevated in response to the tone compared to pre-tone values, but there were no effect of exercise or diet. In the post-tone period, VWR again exhibited significantly ($F_{1,38}$=5.1, p=0.03) higher freezing when compared to sedentary mice. Again, there was no effect of a diet supplemented with EGCG and β-ala.

Hippocampal Cell Proliferation. Analysis of $BrdU^+$ cells in the hippocampal granular cell layer revealed that while the experimental diet had no effect ($F_{1,50}$=0.01, p=0.91), VWR significantly ($F_{1,50}$=16.2, p=0.001) increased (about 2-fold) the total number of $BrdU^+$ cells (**Figure 3.5**).

Hippocampal Gene Expression. The expression of mRNA for a number of neurotrophins, cytokines and chemokines known to be related to learning/memory, neurogenesis or age-related

dysfunction (**Table 3.4**) was measured. With respect to neurotrophins, VWR significantly ($F_{1,36}$=7.1; p=0.01) increased hippocampal Bdnf gene expression. There was no diet or VWR effect on hippocampal Igf1, Ngf, Vegfa or Tgfb1 gene expression. VWR significantly reduced hippocampal expression of two cytokines, Il1β ($F_{1,35}$=6.8; p=0.01) and Itgam/CD11b ($F_{1,37}$=12.4; p=0.001), while the experimental diet tended ($F_{1,38}$=3.2; p=0.08) to reduce Tnf, Il6 mRNA was unchanged in response to any treatment. Of the chemokines, the EGCG/β-ala reduced Cx3cl1 ($F_{1,38}$=4.1; p=0.05), however there were no treatment effects or interactions for Ccl2 or Cxcl12 expression.

***Oxidative Stress in the Brain*.** 4-HNE was measured as a marker for oxidative stress in the cerebellum. Interestingly, diet containing EGCG and β-ala, but not VWR, significantly ($F_{1,35}$=7.5, p=0.01 for diet main effect) reduced oxidative stress (**Figure 3.6**).

3.4 Discussion

We hypothesized that a 4 week administration of a diet containing EGCG and β-ala would lead to additive or synergistic improvements in learning and memory when combined with exercise in aged mice. Thus, our goal was to *improve* age-related cognitive decline in old mice using exercise and targeted dietary supplementation. The rationale for this hypothesis was based upon findings that similar duration interventions of *either* VWR or EGCG administration have been found to improve learning and memory either in normal aging or in models of cognitive loss in rodents. For example, van Praag et al (2005) found that 35 days of VWR (~3.9km/d) significantly improved MWM performance and hippocampal neurogenesis in 19 month old male C57Bl/6 mice. Moreover, EGCG administered i.p. at a dose of 100 mg/kg/d decreased the impairment in learning and memory in spontaneously hypertensive rats (Wang et al., 2012). B-ala was included because it has been shown to improve muscle function and

decrease fatigue (Artioli et al., 2010) and thus might indirectly (via increasing exercise capacity) improve cognition. In addition, a more direct role for β-ala has been suggested in the retrieval of spatial memory because hippocampal β-ala increases after a MWM probe trial (Sase et al., 2013). Thus, the combination of diet and exercise suggests that their effects on the brain can be complimentary and enhance the overall effect on hippocampal molecular measures and cognitive performance.

Our results clearly support a role for exercise in improving age-related reductions in behavioral performance in a one month time frame. We found that VWR mice swam faster in the water maze and therefore were able to get to the hidden platform faster. However, it is not certain that VWR aged mice in our study displayed better spatial abilities on the water maze, as the pathlength to the platform was similar to sedentary animals (**Figure 3.2a**). Although the greater number of crossings through the platform could be interpreted as better spatial memory (**Figure 3.3a and b**), it also is consistent with faster swimming. It is notable that C57BL/6J do not behave this way (van Praag, 2005). More work with BALB/cByJ using other measures of spatial learning such as the Barnes maze, radial-arm maze, T-maze will be necessary to confirm spatial cognition benefits from exercise in BALB/cByJ. Pro-cognitive effects of exercise were much clearer in contextual and cued fear conditioning. VWR mice exhibited elevated behavioral freezing in the original context test of the CFC (**Figure 3.4a**) and in a novel context (**Figure 3.4b**). These findings support a growing literature demonstrating that either long-term (e.g. months) or short-term (e.g. weeks) exercise can improve associative learning in adult and aged animals (van Praag, 2009).

The pro-cognitive effects of exercise are likely mediated by a complex microenvironment that includes increased hippocampal neurogenesis, increased BDNF, IGF-1, FGF-2, EGF,

increased vasculature, glial status, inflammation, neurotransmitter levels, energy metabolism, biochemistry, among many other factors (Cotman et al, 2007). Consistent with this, VWR mice in our study exhibited significantly higher hippocampal BDNF mRNA expression (**Table 3.4**) and $BrdU^+$ cell number in the granular layer of the dentate gyrus (**Figure 3.5**). VWR also led to a reduction in hippocampal IL-1β (a powerful pro-inflammatory cytokine) and ITGAM (CD11b, a microglial inflammatory marker) mRNA expression (**Table 3.4**) indicating an anti-inflammatory effect. In this study, we measured a number or pro-inflammatory cytokines and chemokines because aging is associated with a chronic inflammatory state (e.g. 'inflammaging') (Franceschi et al., 2007) and exercise and EGCG have documented anti-inflammatory effects (Gleeson et al., 2011; Gonzalez et al., 2011). In the context of the brain, IL-1β expression down-regulates BDNF in the hippocampus causing learning and memory disturbances and exercise appears able to counteract these negative effects of inflammation (Barrientos et al., 2011).

Our results do not support a role for short-term (e.g. 4wk) feeding of EGCG and β-ala with or without exercise as a means of improving cognition in the aged. However, we do not want to suggest that these two nutrients would have no effect on cognition if administered over longer durations, at different doses, levels of bioavailability, or as a preventative strategy starting at an earlier age. We observed no independent or additive/synergistic effects of the EGCG/β-ala diet on performance in the MWM (**Figures 3.2-3.3**) or CFC (**Figure 3.4**) tests. We also found no effect of the experimental diet on hippocampal BDNF mRNA (or other neurotrophins), inflammatory cytokines or chemokines (**Table 3.4**), or number of $BrdU^+$ cells as marker for hippocampal cell proliferation (**Figure 3.5**). Taken together, these findings provide strong evidence of a lack of effect of EGCG and β-ala given over the course of a 4 week period on age-related behavioral learning or hippocampal cell proliferation in mice. Interestingly, the diet was

effective at reducing 4-HNE (a by-product of lipid peroxidation) in the cerebellum (**Figure 3.6**), but there was no diet x exercise interaction. EGCG has been reported to have direct anti-oxidant properties as strong radical scavengers and metal chelators *in vitro* and indirect anti-oxidant properties by inducing anti-oxidant enzymes (perhaps by acting as a low level oxidant) such as superoxide dismutase, catalase, and enzymes related to glutathione metabolism in both animal models and humans (Lambert Elias 2010). While we did not see any treatment-induced changes in gene expression for superoxide dismutase 2 (SOD2; mitochondrial manganese SOD) in the hippocampus (data not shown), we did not assess other endogenous anti-oxidant genes or their activity. While there is some controversy as to whether β-ala acts as an anti-oxidant, carnosine (which increases in tissues after β-ala supplementation) scavenges both reactive oxygen and nitrogen species (Budzen et al., 2013). These properties could explain the reduction seen in 4-HNE in this study.

We can offer several possible explanations as to why the diet containing EGCG and β-ala failed to improve existing age-related cognitive deficits as hypothesized. First, our dosages of EGCG and β-ala may have been too low. Our strategy for dosing was to administer a high oral dose, but with enough of a safety factor that adverse effects would not be exhibited. It has been demonstrated that high oral doses of EGCG >500 mg/kg/d cause hepatotoxicity and mortality (Lambert et al., 2010) and doses >1000 mg/kg/d increase *ex vivo* pro-inflammatory cytokine production (Pae et al., 2012) in mice. Moreover, high (>800 mg) oral doses of β-ala can cause paresthesia in humans (Decombaz, et al., 2011) and doses >~5000mg/kg/day can cause taurine depletion in rodents (Murakami et al., 2010). We chose our dosages of EGCG and β-ala based upon prior published studies in rodents and humans that have documented beneficial effects on brain and muscle function (Everaert et al., 2013; Li et al., 2009; Li et al., 2010). For example, Li

et al (2009) found that 6 months of 160 mg/kg/d of green tea catechins could prevent age-related spatial learning and memory decline in C57BL/6 mice. As such, mice in our study consumed on average 182.4 mg/kg/d of EGCG and 417 mg/kg/d of β-ala. For EGCG, the human dose-equivalent for a 70 kg person would be about 60-70 cups of green tea/d (150-180 mg of EGCG per cup of green tea); a dose that could be achievable in people taking EGCG supplements. We feel that our dosages were high enough to see an effect if one existed and low enough to be confident that detrimental side effects did not occur.

We observed no signs or symptoms of illness or altered behavior in our EGCG/β-ala-treated mice. There were no significant differences in food disappearance, body or spleen weight, or running distance between the experimental and control diet groups (**Table 3.3**). The treated animals also displayed no observable behavioral sickness phenotype (e.g. ruffled fur, hunched posture, lethargy) and no difference in swim velocity on the MWM when compared to control-fed mice. Interestingly, water disappearance was statistically greater (~15%) in the EGCG/β-ala-treated mice compared to controls (**Table 3.3**). To our knowledge, this phenomena has not been reported in the EGCG or β-ala literature. In fact, higher dose 3% β-ala intake in drinking water has been reported to reduce fluid and sodium excretion and reduce fluid intake (Mozaffari, et al., 1997). Lastly, it could be that the length of our feeding regimen was too short to cause beneficial changes in behavioral learning in these aged mice. Indeed, Li et al 2009 demonstrated that long-term (6 months from 14-20 months of age) feeding (in drinking water) of green tea catechins dose-dependently prevented age-related loss of spatial learning by increasing CREB phosphorylation and BDNF and Bcl-2 expression in C57BL/6J mice (Li et al., 2009). Their high dose corresponded to ~160 mg/kg/d which was similar to our dosage (e.g. 182 mg/kg/d). It was encouraging in our study that the experimental diet reduced oxidative stress in the brain

suggesting that a longer period of oxidative protection may be needed to affect learning, memory and hippocampal neurogenesis. Future studies should examine the effect of long-term EGCG supplementation with exercise to determine if there is an additive or synergistic effect on prevention of memory loss; clearly in our study this short-term regimen was unable to alter age-related cognitive loss.

Studies on the bioavailability of green tea catechins like EGCG yield different results. Catechins are conjugated in enterocytes and methylated, sulfated and glucuronidated in the liver (Manach et al., 2004). Fractions of catechins not absorbed in the small intestine are acted upon by the gut microbiota forming smaller molecules (Del rio et al., 2010). Nonetheless, a fraction of ingested catechins do reach the systemic circulation in humans (Sherry-Chow et al., 2003) and mice (Lambert et al., 2003). Access of catechins to the brain is also a matter of debate. One *in vivo* study demonstrated that, while flavonols or their metabolites appear in the circulation, they do not cross the blood brain barrier (Zini et al., 2006). This is in contrast to *in vitro* experiments demonstrating that such molecules can cross endothelial cell barriers (Faria et al., 2011) and to a study in rats that found that low levels of EGCG and its metabolites could be detected in several brain regions following oral administration of 100 mg/kg of EGCG. β-ala is much more bioavailable and ingestion has been robustly found to increase β-ala and, importantly, carnosine in muscle and brain (Derave et al., 2010; Murakami et al., 2010).

In conclusion, we have demonstrated that 4 weeks of voluntary wheel running, but not a diet containing EGCG and β-ala, enhanced associative memory, increased hippocampal cell proliferation and BDNF expression, while reducing inflammation in aged Balb/cByJ mice. Future research is needed to examine whether the dosages of EGCG and β-ala were sufficient to increase brain EGCG and/or carnosine levels. Likewise, the impact of long-term feeding in

conjunction with exercise should be explored as a strategy to prevent age-related cognitive loss. Our data suggest that short-term feeding of EGCG and β-ala, at fairly high oral dosages, do not independently or synergistically act with exercise to reverse age-related cognitive loss.

3.5 Figures and Tables

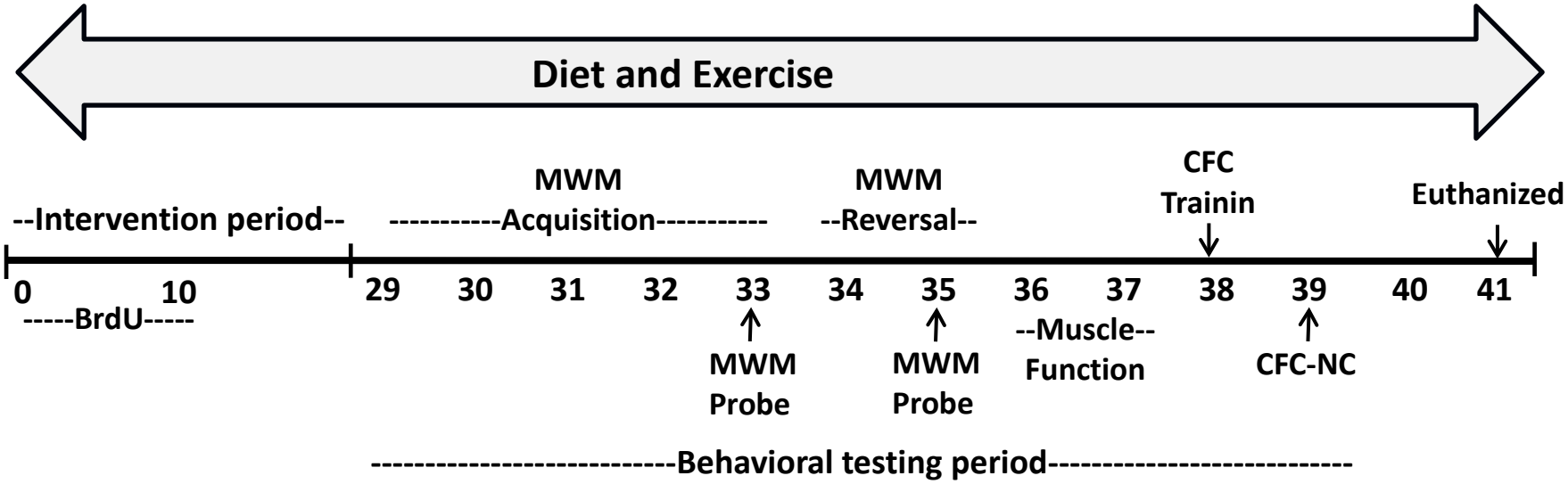

Figure 3.1. Experimental timeline. Experimental intervention (dietary and exercise) continued through duration of study.
Morris water maze (MWM).
Contextual fear conditioning (CFC).
Novel Context (NC).

Component		Con	EGCG/β-ala
Protein	(% by wt.)	14	14
Carbohydrate	“	73	73
Fat	“	4	4
Energy	(kcal/g)	3.8	3.8
Casein	(g/kg)	140	140
L-Cystine		1.8	1.8
Corn Starch	“	496	496
Maltodextrin 10	“	125	125
Sucrose	“	100	100
Cellulose, BW 200	“	50	50
Soybean Oil	“	40	40
tButylhydroquinone	“	0.008	0.008
Mineral Mix S10022M	“	35	35
Vitamin Mix V10037	“	10	10
Choline Bitartrate	“	2.5	2.5
Blue Dye #1	“	—	0.05
Teavigo (EGCG)	(mg/g)	0	1.5
β-ala	“	0	3.43

Table 3.1. Diet (AIN-93M) composition.

Gene	Classification	Assay Identification[a]
Bdnf	Neurotrophin	Mm01334042_m1
Ngf	Neurotrophin	Mm00443039_m1
Igf1	Neurotrophin	Mm00439560_m1
Vegfa	Neurotrophin	Mm01281449_m1
Tgfb1	Neurotrophin	Mm01178820_m1
Tnf	Pro-inflammatory	Mm00443258_m1
Il1β	Pro-inflammatory	Mm00434228_m1
Il6	Pro-Inflammatory	Mm00446190_m1
Itgam (Cd11b)	Pro-Inflammatory	Mm00434455_m1
Ccl2	Chemokine	Mm00441242_m1
Cx3cl1	Chemokine	Mm00436454_m1
Cxcl12	Chemokine	Mm00445553_m1

Table 3.2. Quantitative real-time PCR primer information.
[a]Applied Biosystems TaqMan Gene Expression Assay identification number.

Variable	Con-Sed	EGCG/β-ala-Sed	Con-VWR	EGCG/β-ala-VWR
day -28 Body Weight (g)	31.1 ± 0.5	30.3 ± 0.6	30.5 ± 0.6	30.7 ± 0.5
day 13 Body Weight (g)	30.0 ± 0.6	29.1 ± 0.6	27.0 ± 0.5 *	27.5 ± 0.5 *
Food Disappearance (g/day)	3.4 ± 0.1	3.5 ± 0.1	3.6 ± 0.1	3.6 ± 0.1
Water Disappearance (g/day)	2.7 ± 0.2	3.4 ± 0.2 ^	3.1 ± 0.2	3.3 ± 0.2 ^
Spleen Weight (mg)	94 ± 5	88 ± 7	89 ± 9	81 ± 7
Running Distance (km/day)			4.8 ± 0.8	4.5 ± 0.4

Table 3.3 Descriptive Data. Data are shown as the mean ± SEM. *$p < 0.05$ for the main effect of VWR; ^$p < 0.05$ for the main effect of diet; n= 13, 15, 15, and 15 for Con Sed, EGCG/β-ala Sed, Con VWR, and EGCG/β-ala VWR, respectively.

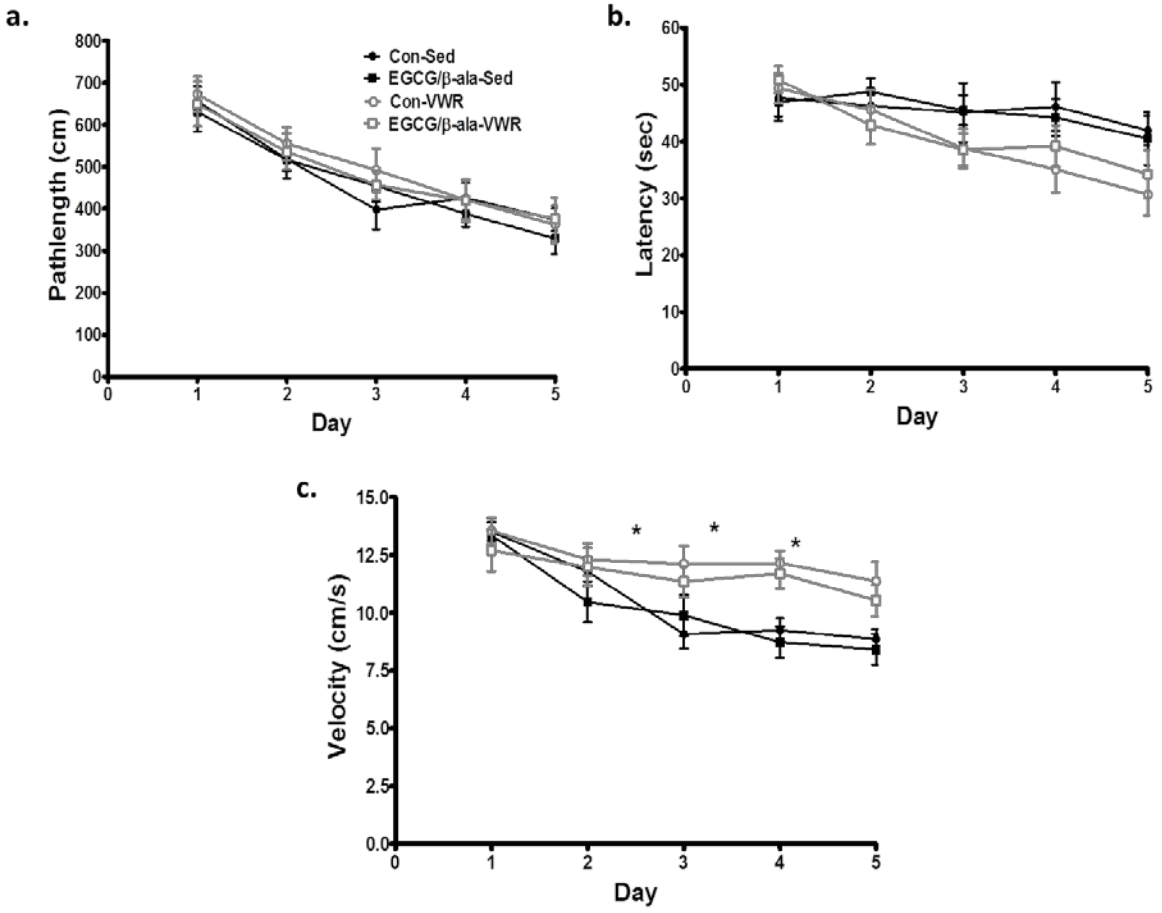

Figure 3.2. Exercise, but not EGCG/β-ala, decreases latency during a test of spatial learning of aged mice in the Morris water maze. All mice learned the task as evidence by a significantly shorter pathlength to the hidden platform over time (a). VWR mice found the platform faster later in the acquisition period (b), this effect was most probably due to significantly faster swim speeds (c). Data are shown as the mean ± SEM. *$p < 0.05$ for the main effect of exercise; n = 12, 14, 15 and 15 for Con Sed, EGCG/β-ala Sed, Con VWR, and EGCG/β-ala VWR, respectively.

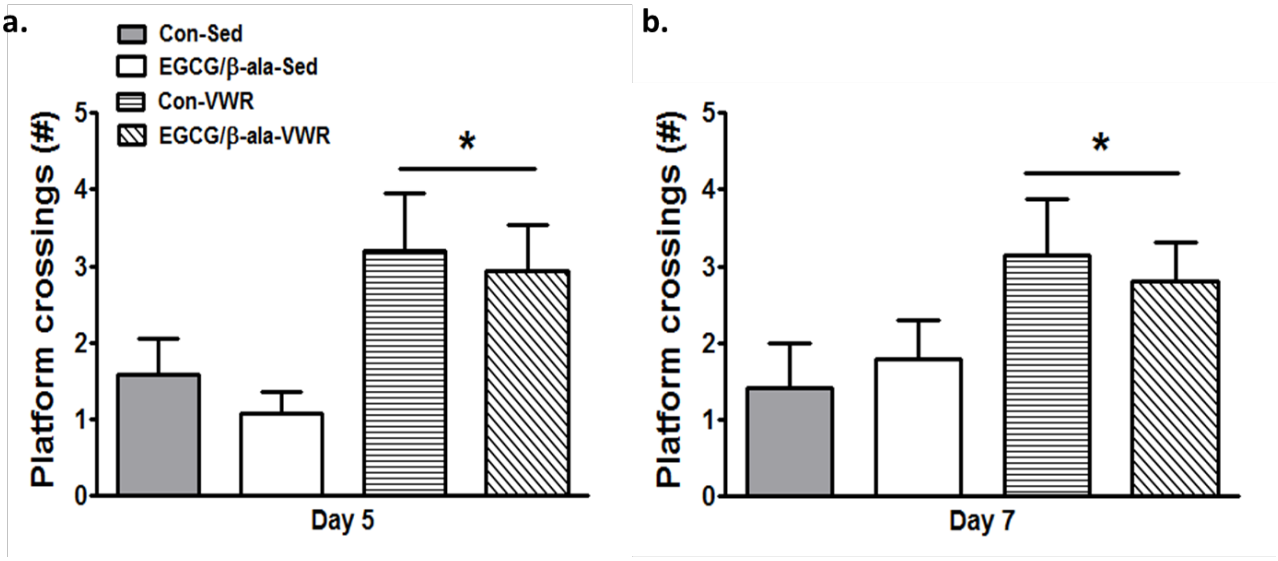

Figure 3.3. Exercise, but not EGCG/β-ala, enhances spatial preference for platform location during probe trials in the Morris water maze. On both Day 5 (a) and 7 (b), VWR mice crossed area where platform was located more often than sedentary mice. Data are shown as the mean ± SEM. *$p<0.05$ for the main effect of exercise; n= 12, 13, 14, and 15 for Con Sed, EGCG/β-ala Sed, Con VWR, and EGCG/β-ala VWR, respectively.

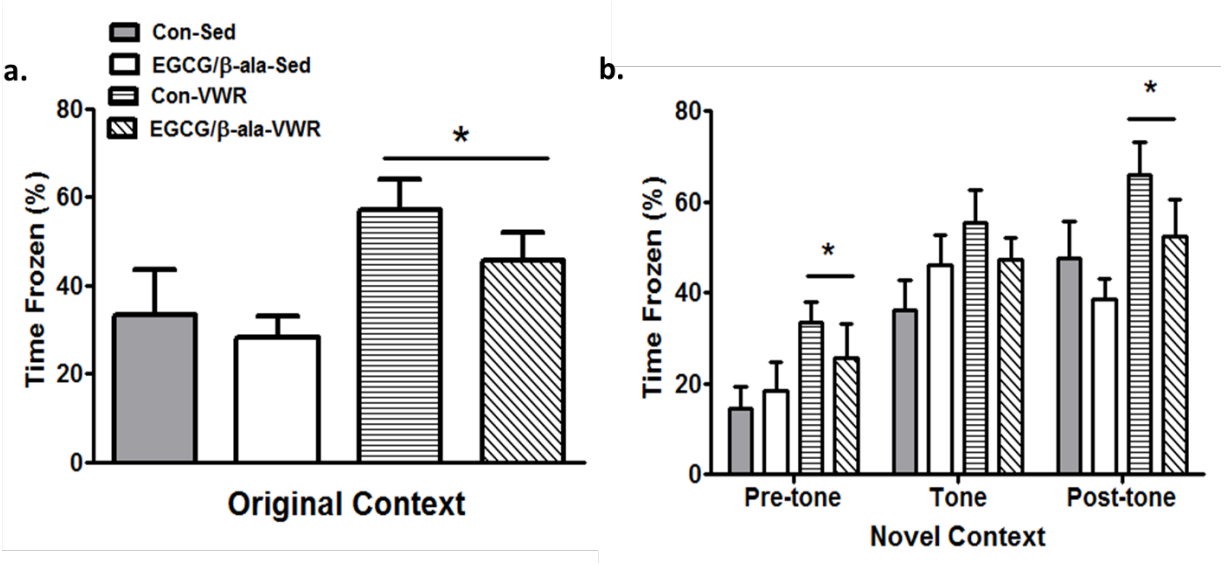

Figure 3.4. Exercise, but not EGCG/β-ala, improves learning in the contextual fear conditioning test. In pre-conditioned animals, VWR resulted in a significant increase in time spent frozen when re-introduced into the original context 24 hrs later when compared to sedentary aged mice (a). In the novel context (5b), VWR increased freezing pre- and post-tone conditions. There were no significant group differences in the tone condition. Data are shown as the mean ± SEM. *$p < 0.05$ for the main effect of exercise; n = 10, 11, 11, and 10 for Con Sed, EGCG/β-ala Sed, Con VWR, and EGCG/β-ala VWR, respectively.

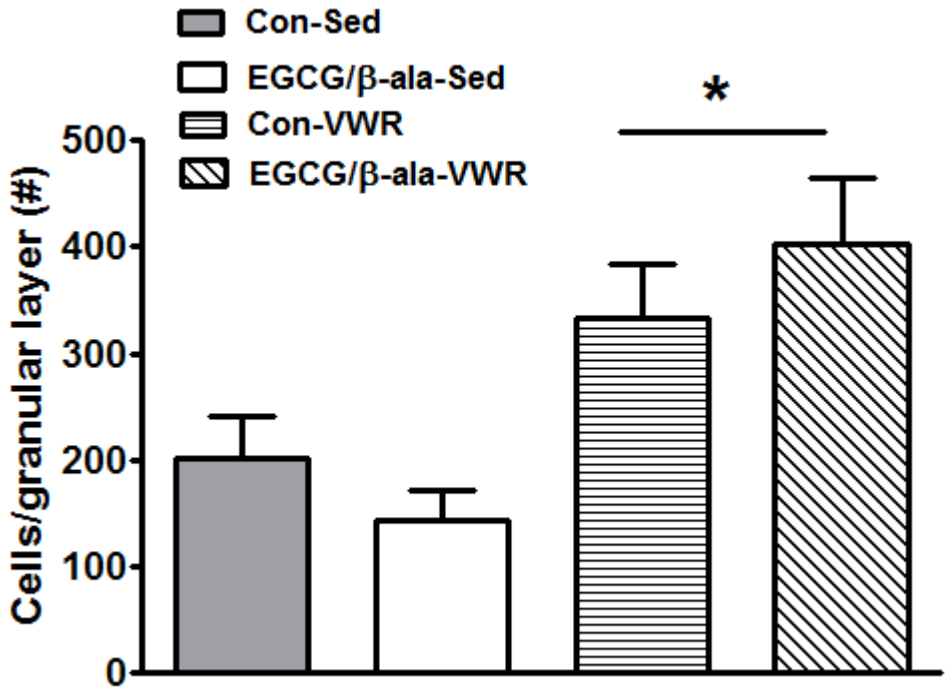

Figure 3.5. VWR, but not EGCG/β-ala, increase hippocampal cell proliferation. Data are shown as the mean ± SEM. *$p < 0.05$ for the main effect of exercise; n = 12, 13, 14, and 15 for Con Sed, EGCG/β-ala Sed, Con VWR, and EGCG/β-ala VWR, respectively.

	Group				Statistics (p values)		
Gene	**Con-SED**	**EGCG β-ala -SED**	**Con-VWR**	**EGCG β-ala -VWR**	**Diet**	**VWR**	**Diet x VWR**
Neurotrophins							
Bdnf	1.00 ± 0.09	0.92 ± 0.06	1.11 ± 0.06	1.16 ± 0.09	0.86	**0.03**[a]	0.41
Ngf	1.00 ± 0.08	1.00 ± 0.08	1.05 ± 0.04	0.96 ± 0.05	0.49	1.00	0.45
Igf1	1.00 ± 0.07	1.19 ± 0.10	1.13 ± 0.07	1.12 ± 0.08	0.28	0.68	0.24
Vegfa	1.00 ± 0.05	0.97 ± 0.09	1.00 ± 0.09	0.86 ± 0.07	0.54	0.25	0.47
Tgfb1	1.00 ± 0.08	1.01 ± 0.09	1.13 ± 0.07	1.01 ± 0.07	0.40	0.49	0.44
Pro-inflammatory							
Tnf	1.00 ± 0.17	0.86 ± 0.13	0.99 ± 0.10	0.73 ± 0.10	0.12	0.61	0.65
Il1β	1.00 ± 0.11	0.84 ± 0.10	0.68 ± 0.08	0.73 ± 0.10	0.60	**0.04**[a]	0.27
Il6	1.00 ± 0.14	1.10 ± 0.13	1.33 ± 0.17	1.24 ± 0.20	0.97	0.18	0.55
Itgam (Cd11b)	1.00 ± 0.05	0.95 ± 0.07	0.82 ± 0.04	0.77 ± 0.04	0.34	**0.001**[a]	0.98
Chemokines							
Ccl2	1.00 ± 0.14	0.87 ± 0.13	0.96 ± 0.14	0.96 ± 0.22	0.67	0.87	0.67
Cx3cl1	1.00 ± 0.03	0.87 ± 0.05	1.01 ± 0.04	0.94 ± 0.04	**0.03**[a]	0.39	0.55
Cxcl12	1.00 ± 0.04	1.02 ± 0.10	1.03 ± 0.06	0.86 ± 0.05	0.26	0.33	0.15

Table 3.4. Hippocampal gene expression following 39 days of dietary and VWR treatment. Values are reported relative to Con-SED using ΔΔCT method and GAPDH as the control gene. n = 8-12 /treatment.

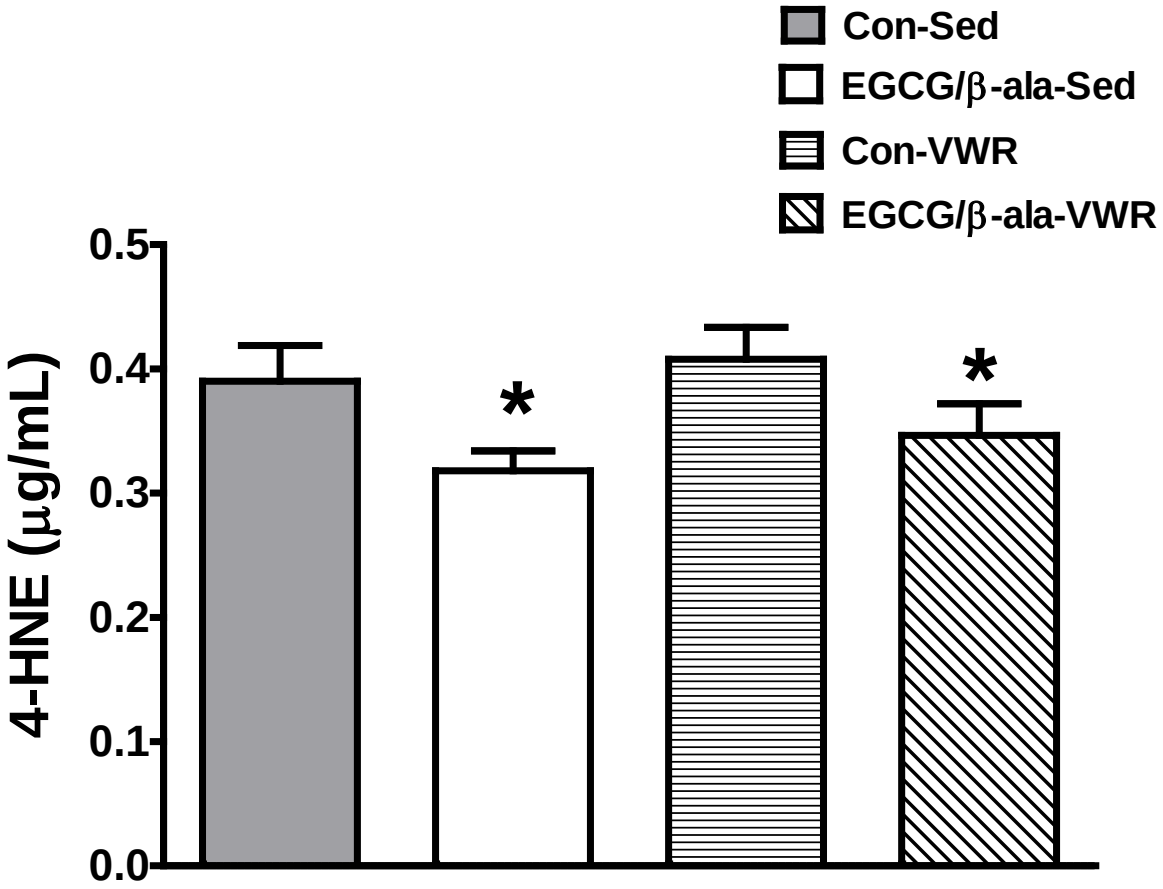

Figure 3.6. A diet containing EGCG and β-ala reduces 4-HNE in the cerebellum of aged mice. Data are represented as means ± SEM. *$p < 0.05$ for the main effect of diet; n = 9, 10, 9, and 11 for Con Sed, EGCG/β-ala Sed, Con VWR, and EGCG/β-ala VWR, respectively.

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Chapter 4

Summary and Future Considerations

The proportion of adults over 65 years of age is expected to increase to 1.5 billion people over the next 40 years and mostly in developing countries (2011). Aging is associated with an increased risk of developing debilitating chronic diseases, specifically those related to cognitive impairment. Even the "healthy" aging can experience age-related cognitive decline. As the brain ages, there is an increase in inflammation, oxidative stress, and a reduction in neurotrophins. Our lab and others have demonstrated that moderate increases in pro-inflammatory cytokine production in the brain of aged animals plays a critical role in the decrease of new cell proliferation and cognition (Barrientos et al., 2010; Blackmore et al., 2009; Buchanan et al., 2008; Godbout et al., 2005; Jang et al., 2010; Kohman et al., 2012). Thus, research for preventing or treating age-related cognitive decline is vital for investigating new and cost-effective ways to impact the negative aspects of aging.

In recent years, the number of publications centered on aging, neurodegeneration, and alternative therapeutic interventions has steadily increased. With this has come a new focus on characterizing neuromodulatory pathways, including the role of microglia cells in aging and cognition. Additionally, the impact of exercise and dietary supplements on aging and cognitive function has been pushed to the forefront. The dysregulation of pro- and anti-inflammatory homeostasis in the aged brain and how this dysregulation can alter new cell proliferation and cognition is of particular interest.

Increases in pro-inflammatory cytokines, oxidative stress, and decreases in new cell proliferation have been strongly correlated with aging and development of neurodegenerative

diseases, such as AD and PD (Hirsch and Hunot, 2009; Lazarov et al., 2005). Interest in finding therapeutics alternatives to prevent, delay and treat cognitive disorders associated with normal aging and/or neurodegenerative disease is gaining attention. Indeed, there is a lot of literature about the benefits of physical activity on brain health and cognition in the aged population and multiple studies have examined the benefits of EGCG for learning and memory in the aged. More recently, β-ala has been suggested to have cognitive benefits. Limited evidence exists on how dietary supplements and exercise can synergize or have an additive effect to improve learning and memory in normal aged mice.

The data presented indicate that exercise increases new cell proliferation in the dentate gyrus of the hippocampus and that this production of new cells can be associated with improved performance during hippocampal-dependent learning and memory tasks as shown with the Morris water maze and contextual fear conditioning paradigm. Furthermore, exercise led to a significant increase in BDNF expression while reducing the inflammatory response in aged mice compared to sedentary controls. Diet supplemented with EGCG and β-ala did not significantly alter new cell proliferation or gene expression within the hippocampus, but did significantly reduce 4-HNE (a by-product of lipid peroxidation) in the cerebellum of aged mice relative to control diet mice. Thus, future investigation should focus on the antioxidant roles of EGCG and β-ala as well as carnosine in the brain. Moreover, due to the low bioavailability of EGCG and the abundant expression of β-ala/carnosine in tissue, further research is needed to examine if the dosage and duration of the supplemented diet were sufficient to increase levels of EGCG and β-ala/carnosine in brain and muscle tissue. Additional studies examining the protective effects of dietary supplementation and exercise on an immune challenge in the aged is warranted. Neuroinflammation in aged animals may be too modest to elicit dietary effects. The aged brain is

more vulnerable to disruptive effects of extrinsic factors such as disease, infection, or stress. Therefore, a LPS challenge will create an elevated inflammatory response in which dietary supplementation may improve molecular markers associated with inflammation and further enhance exercise related improvements in age-related cognitive decline.

4.1 References

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